09/980364

Rec'd PCT/PTO 30 NOV 2001

USE OF THE *BNM3* TRANSCRIPTIONAL ACTIVATOR TO CONTROL PLANT EMBRYOGENESIS AND REGENERATION PROCESSES

BACKGROUND OF THE INVENTION

A typical angiosperm seed consists of three major components, the embryo, the endosperm and the maternal seed coat. Seed development begins with a double fertilization event, in which one sperm cell nucleus fuses with the egg cell nucleus to form the embryo, and a second sperm cell nucleus fuses with two central cell nuclei to form the endosperm. Embryo development itself can be separated into three developmental phases. The first phase of embryo development is one of cell division and morphogenesis, which serves to establish the major tissue types and organ systems of the mature plant. The second phase encompasses a period of rapid cell expansion and is characterized by the synthesis of storage reserves that sustain the embryo during germination and early seedling development. In the final phase of embryo development, the embryo becomes desiccated and enters into a period of developmental arrest or dormancy. All of the above events normally take place while the seed remains attached to the maternal plant.

Many plant species are capable of producing embryos in the absence of fertilization. This process of asexual embryo development may occur naturally, for example on the leaf margins of *Bryophyllum* (Yarborough, 1923) and *Malaxis* (Taylor, 1967), or within the ovule of apomictic plants (Koltunow, 1995). Apomixis refers to the production of a seed from the maternal ovule tissues in the absence of egg cell fertilization. Asexual embryo development may also be induced *in vitro* from gametophytic or somatic tissue (Mordhorst *et al.*, 1997) or, as shown recently, may be induced by genetic modification of gene expression (Ogas *et al.*, 1997; Lotan *et al.*, 1998).

Three major mechanisms of apomixis, diplospory, apospory and adventitious embryony, have been observed. Each mechanism differs with respect to the source of the cell that gives rise to the embryo and with respect to the time during ovule development at which the apomictic process is initiated. Diplospory and apospory are considered gametophytic forms of apomixis as

they involve the formation of diploid embryo sacs. Adventitious embryony does not involve the production of a mitotically-derived embryo sac.

In diplospory, the megaspore mother cell does not undergo normal meiosis, but rather divides mitotically to produce a diploid embryo sac instead of the normal haploid embryo sac. One of the cells of the embryo sac functions as the egg cell and divides parthenogenetically (without fertilization) to form an embryo. In some species the unreduced polar nuclei of the embryo sac may fuse to form the endosperm (autonomous endosperm production), the nutritive tissue of the seed, while in other species pollination is necessary for endosperm production (pseudogamy).

In aposporous apomicts, parthenogenic embryos are produced from additional cells, the aposporous initials, that differentiate from the nucellus. As with the megagametophyte of diplosporous species, the aposporous initial undergoes mitotic divisions to produce a diploid embryo sac. Aposporous embryos are not derived from the megagametophyte and can therefore co-exist within a single ovule with sexually-derived embryos. Autonomous production of endosperm is rare in aposporous species. Aposporous apomicts therefore depend on fertilization of the polar nuclei of a meiotically-derived embryo sac for the production of endosperm.

With adventitious embryony, embryos are formed directly from sporophytic ovule tissue, such as the integuments or nucellus, via parthenogenesis. Seeds derived from species exhibiting adventitious embryony generally contain multiple asexually-derived embryos and may also contain a single sexually-derived embryo. Plants exhibiting adventitious embryo also rely on the presence of a meiotically-derived embryo sac within the same ovule for endosperm formation.

In most plant species, the apomictic trait appears to be under the control of a single dominant locus. This locus may encode one or more developmental regulators, such as transcription factors, that in sexually reproducing plants function to initiate gene expression cascades leading to embryo sac and/or embryogenesis, but which are heterochronically or ectopically expressed in apomictic plants (Peacock, 1995; Koltunow, 1993; Koltunow et al, 1995).

Apomixis is a valuable trait for crop improvement since apomictic seeds give rise to clonal offspring and can therefore be used to genetically fix hybrid lines. The production of hybrid seed is a labour intensive and costly procedure as it involves maintaining populations of genetically pure parental lines, the use of separate pollen donor and male-sterile lines, and line isolation. Production of seed through apomixis avoids these problems in that once a hybrid has been produced, it can be maintained clonally, thereby eliminating the need to maintain and cross separate parental lines. The use of apomictic seed also provides a more cost effective method of multiplying vegetatively-propagated crops, as it eliminates the use of cuttings or tissue culture techniques to propagate lines, reduces the spread of diseases which are easily transmitted through vegetatively-propagated tissues, and in many species reduces the size of the propagule leading to lower shipping and planting costs.

Although apomixis occurs in a wide range of plant species, few crop species are apomictic. Attempts to introduce apomictic traits into crop species by introgression from wild relatives (Ozias-Akins, *et al.*, 1993; WO 97/10704; WO 97/11167) or through crosses between related, but developmentally divergent sexual species (WO 98/33374), have not yielded marketable products. Other approaches have focused on the identification of gene sequences that may be used to identify or manipulate apomictic processes (WO 97/43427; WO 98/36090), however these approaches have not led to methods for the routine production of apomictic plants.

Mutagenesis approaches have also been attempted to convert sexually reproducing plants such as *Arabidopsis thaliana* (arabidopsis) into apomictic plants (Peacock *et al.*, 1995). For example, a number of recessive "fertilization-independent seed" (*fis*) mutants have been identified that initiate partial embryo and/or endosperm at a low frequency in the absence of fertilization (Chaudhury *et al.*, 1997). However, a number of additional parameters need to be modified in order to obtain true diploid apomictic seed using *fis* mutants.

Asexually-derived embryos can be induced to form in culture from many gametophytic and somatic plant tissues (Yeung, 1995). Somatic embryos can be obtained from culture of somatic tissues by treating them with plant growth regulators, such as auxins, or auxins in

combination with cytokinins. Embryos can also be induced to form in culture from the gametophytic tissues of the ovule (gynogenesis) and the anther (androgenesis, pollen or microspore embryogenesis), either by the addition of plant growth regulators or by a simple stress treatment.

Several mutants have been identified that may be used to induce efficient production of embryos *in vitro*. These include recessive arabidopsis mutants with altered shoot meristems, for example *primordia timing (pt)*, *clavata (clv)1* and *clv3*, which were shown to enhance embryogenic callus formation when seedlings were germinated in the presence of auxin (Mordhorst *et al*., 1998). The altered expression of two arabidopsis genes, *LEAFY COTYLEDON* (*LEC1*; WO 98/37184, Lotan *et al*., 1998) and *pickle*, have been shown to promote the production of somatic embryos in the absence of added growth regulators. The *LEC1* gene encodes a homologue of the HAP3 subunit of a CCAAT box-binding transcription factor (CBF). The *LEC1* gene controls many aspects of zygotic embryo development including desiccation tolerance and cotyledon identity. Ectopic over-expression of the *LEC1* gene in a *lec1* mutant background results in the production of 2 transgenic lines that occasionally form embryo-like structures on leaves. These embryo-like structures express genes, such as those encoding seed storage proteins and oil body proteins, which are normally preferentially expressed in developing embryos. Plants containing a recessive mutant *PICKLE* gene produce a thickened, primary root meristem. Mutant *pickle* roots produce embryo-forming callus when the root tissue is separated from the rest of the plant and placed on minimal medium without growth regulators (Ogas *et al*., 1997). Mutant *pickle* roots show morphological characteristics of developing seeds, such as oil bodies and, as with *LEC1* over-expressers, accumulate genes preferentially expressed in developing seeds.

Efficient production of apomictic seed is only likely to be realised through the identification and subsequent modification of developmental regulators, such as transcription factors, that are known to activate gene expression cascades leading to embryogenesis in both sexually-reproducing and apomictic plants. The present invention addresses this need by providing methods for the production of apomictic seeds comprising ectopic over-expression of an embryo-expressed AP2 domain containing transcription factor, BNM3 or its homologs.

It is an object of the invention to overcome disadvantages of the prior art.

The above object is met by the combinations of features of the main claims, the sub-claims disclose further advantageous embodiments of the invention.

SUMMARY OF THE INVENTION

The present invention relates to asexual embryo formation and regeneration in plants. More specifically, it relates to processes for producing asexually-derived embryos, and for enhancing regeneration capacity in plants.

According to the present invention there is provided an isolated DNA molecule comprising a nucleotide sequence that:

- hybridizes to SEQ ID NO:5 or 6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or stringent conditions;
- hybridizes to SEQ ID NO:1 or 3, not including the AP2 repeat1-linker-AP2 repeat2 region, under stringent conditions;
- comprises at least 27 contiguous nucleotides of SEQ ID NO's:1, 3, 5 or 6; or that
- exhibits at least 70% similarity with the nucleotide sequence defined by SEQ ID NO's:1, 3, 5 or 6.

This invention further relates to an isolated DNA molecule that hybridizes to SEQ ID NO's: 1, 3, 5 or 6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or stringent conditions, and comprises a nucleic acid sequence encoding a protein, wherein the protein when present at a sufficient level within a plant cell renders the cell embryogenic, increases the regenerative capacity of the plant cell, or both renders the cell embryogenic and increases the regenerative capacity of the plant cell. Included within the present invention is the isolated DNA molecule as just defined comprising a nucleotide sequence that hybridizes to nucleotides 1-2014 of SEQ IDNO:1, 1-2011 of SEQ ID NO:3, 1620-4873 of SEQ ID NO:5, or nucleotides 2026-5035 of SEQ ID NO:6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or stringent conditions. Also included within the present invention is a vector comprising the isolated DNA molecule as defined above, wherein the isolated DNA molecule is under control of a regulatory element that directs expression of said DNA in a plant cell. The regulatory element may be a constitutive, inducible, tissue specific or a developmental active, regulatory element.

This invention also embraces a transformed plant cell, a transformed plant, or seed obtained from a transformed plant, each comprising the vector as defined above

This invention relates to an isolated protein encoded by an isolated DNA molecule that hybridizes to the nucleotide sequence defined by SEQ ID NO:1, 3, 5 or 6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or stringent conditions, wherein the protein, when present at a sufficient level within a plant cell renders the cell embryogenic, or increases the regenerative capacity of the plant cell. Also included is a protein encoded by an isolated DNA molecule that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5, or nucleotides 2026-5035 of SEQ ID NO:6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under moderate or stringent conditions. This invention also embraces an isolated DNA molecule that encodes a protein as defined by SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:7. The invention also pertains to a protein comprising at least 70% homology with the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:7, or comprises from about 30 to about 541 amino acids of the sequence disclosed in SEQ ID NO:2, or comprises from about 30 to about 561 amino acids of the sequence disclosed in SEQ ID NO:4.

The present invention is also directed to a method of producing asexually derived embryos comprising:

i) transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO's: 1, 3, 5, or 6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of the plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5, or nucleotides 2026-5035 of SEQ IDNO:6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under moderate or stringent conditions;

ii) growing the plant cell to produce transformed tissue;

iii) selecting the transformed tissue for occurrence of the isolated DNA molecule; and

iv) assaying the transformed tissue for asexual embryo formation.

This invention also relates to the above method where the step of assaying (step iv)) involves assaying for somatic embryos, gametophytically-derived embryos, adventitious embryony, diplospory, or for haploid parthenogenesis of the embryo sac.

The present invention also embraces a method of producing an apomictic plant comprising:

i) transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to nucleotides 1-2014 of SEQ ID NO:1, nucleotides 1-2011 of SEQ ID NO:3, nucleotides 1620-4873 of SEQ ID NO:5, or nucleotides 2026-5035 of SEQ IDNO:6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or stringent conditions and which encodes a protein that when present at a sufficient level within said plant cell renders the plant cell embryogenic, or increases the regenerative capacity of the plant cell;

ii) selecting the transformed plant for occurrence of the isolated DNA molecule; and

iii) assaying the transformed plant for asexual embryo formation.

This invention also relates to the above method where the step of assaying (step iii)) involves assaying for asexually-derived embryos, somatic embryos, gametophytically-derived embryos, adventitious embryony, diplospory, or for haploid parthenogenesis of the embryo sac.

The present invention is also directed to a method of producing asexually derived embryos comprising:

i) transiently transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to nucleotides 1-2014 of SEQ ID NO:1, nucleotides 1-2011 of SEQ ID NO:3, nucleotides1620-4873 of SEQ ID NO:5, or nucleotides 2026-5035 of SEQ IDNO:6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or under stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of the plant cell;

ii) growing the transiently transformed plant cell to produce transiently transformed tissue;

iii) assaying the transiently transformed tissue for asexual embryo formation.

This invention is directed to the above method where the step of assaying (step iii)) involves assaying for asexually-derived embryos, somatic embryos, gametophytically-derived embryos, adventitius embryony, diplospory, or for haploid parthenogenesis of the embryo sac.

The present invention also presents a method of modifying the regenerative capacity of a plant comprising

i) transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 or SEQ ID NO:6 under moderate or stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of said plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5, or nucleotides 2026-5035 of SEQ IDNO:6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or under stringent conditions, or to the nucleotide sequence as defined within nucleotides 1-2014 of SEQ ID NO:1, or nucleotides 1-2011 of SEQ ID NO:3 under stringent conditions;

ii) growing the transformed plant cell to produce transformed tissue; and

iii) assaying the transformed tissue for enhanced regeneration as compared to wild type tissue.

This invention also embraces the above method wherein step iii) includes assaying in the absence of a growth regulator.

The present invention also relates to a method of modifying the regenerative capacity of a plant comprising;

i) transiently transforming a plant cell with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 or SEQ ID NO:6 under moderate or stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the

regenerative capacity of the plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5, or nucleotides 2026-5035 of SEQ IDNO:6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions;

ii) growing the transiently transformed plant cell to produce transiently transformed tissue;

iii) assaying the transformed tissue for enhanced regeneration as compared to wild type tissue.

This invention also embraces the above method wherein step iii) includes assaying in the absence of a growth regulator.

The present invention also relates to a method of selecting a transformed plant comprising;

i) transforming a normally non-regenerative plant with a vector comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 or SEQ ID NO:6 under moderate stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of said plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620- 4873 of SEQ ID NO:5, or nucleotides 2026-5035 of SEQ IDNO:6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions; and

ii) determining whether the transformed plant is able to regenerate under conditions in which the normally non-regenerative plant does not regenerate.

The present invention is also directed to an isolated DNA molecule comprising a DNA sequence that exhibits at least about 70% similarity with nucleotides 1-1619 of SEQ ID NO:5, or nucleotides 1-2025 of SEQ ID NO:6, or that comprises at least 22 contiguous nucleotides within nucleotides 1-1619 of SEQ ID NO:5 or 1-2025 of SEQ ID NO:6. Also included within

the scope of the present invention is a vector comprising the isolated DNA molecule as just defined, operably associated with a gene of interest, wherein the isolated DNA molecule directs the expression of the gene of interest within a plant cell. The gene of interest may be heterologous with respect to the isolated DNA molecule. The gene of interest may be selected from the group consisting of a pharmaceutically active protein, antibody, industrial enzyme, protein supplement, nutraceutical, storage protein, animal feed and animal feed supplement. This invention also includes a transformed plant cell, a transformed plant, or seed obtained from the transformed plant, comprising the vector as just defined.

Furthermore, the present invention includes a method for directing the expression of a gene of interest within a developing embryo of a plant comprising transforming said plant with a vector containing an isolated DNA molecule that exhibits at least about 70% similarity with nucleotides 1-1619 of SEQ ID NO:5, or nucleotides 1-2025 of SEQ ID NO:6, or that comprises at least 22 contiguous nucleotides within nucleotides 1-1619 of SEQ ID NO:5 or nucleotides 1-2025 of SEQ ID NO:6.

This invention also pertains to a method of producing a protein of interest comprising

i) transforming a plant with at least one vector, comprising an isolated DNA molecule that hybridizes to SEQ ID NO:5 or SEQ ID NO:6 under moderate or stringent conditions and which encodes a protein that when present at a sufficient level within the plant cell renders the plant cell embryogenic, or increases the regenerative capacity of said plant cell, or that comprises a nucleotide sequence that hybridizes to nucleotides 1620-4873 of SEQ ID NO:5, or nucleotides 2026-5035 of SEQ IDNO:6, not including the AP2 repeat1-linker-AP2 repeat2 region, under moderate or under stringent conditions, or to the nucleotide sequence as defined within SEQ ID NO:1 or 3 under stringent conditions to produce a transformed plant;

ii) selecting the transformed plant for occurrence of the isolated DNA molecule; and

iv) growing the transformed plant in order to produce the protein of interest, wherein expression of the protein of interest is induced by the expression product of said isolated DNA.

This method may also comprise transforming the plant with a second vector comprising a nucleotide sequence encoding the protein of interest under the control of a regulatory element, wherein the regulatory element induced by the expression product of the isolated DNA. Furthermore, this method may also be used to produce a protein of interest wherein the protein of interest is a native protein.

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a sub-combination of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

Figure 1 shows a schematic representation of the effect of culture temperature on the developmental fate of isolated microspores and pollen of *Brassica napus*. Late uninucleate microspores and early binucleate pollen cultured at 25 °C or lower continue to divide and form functional pollen grains (gametophytic), while the same microspores and pollen cultured at 32 °C undergo numerous sporophytic divisions, leading to the formation of haploid embryos (embryogenic). Late uninucleate microspores and early binucleate pollen cultured for one day at 25 °C, followed by culture at 32 °C may undergo gametophytic divisions, but form neither embryos nor mature pollen grains (non-embryogenic).

Figure 2 shows the alignment of the DNA sequences depicted in SEQ ID NO:1 and SEQ ID NO:3. The ATG and TAG translation initiation and translation termination codons are shown in bold. Identical nucleotides are indicated by (*) and gaps are indicated by (-).

Figure 3 shows the alignment of the predicted protein sequences encoded by the DNA of SEQ ID NO:1 and SEQ ID NO:3. The amino acid sequence of the first AP2 domain repeat (repeat 1) and the second AP2 domain repeat (repeat 2), are shown in bold. Identical amino acids are indicated by an asterisk (*) and mismatches by a dot (.) below the sequence alignment.

Figure 4 shows the presence of two *BNM3* genes in the *Brassica napus* genome. A DNA gel blot containing restriction digests of *B. napus* c.v. Topas genomic DNA was hybridized to a *BNM3A* cDNA fragment under high stringency conditions. The *BNM3*A cDNA hybridizes to two DNA fragments under these conditions. These fragments correspond to the *BNM3A* and *BNM3B* genes. The position of the molecular size markers (Lambda

DNA *Hind* III restriction fragments) is indicated to the left the figure. The restriction enzymes used to digest the DNA are indicated above the blot.

Figure 5 shows the alignment of the predicted protein sequence encoded by the DNA of SEQ ID NO.1 (BNM3A) with the predicted protein sequences of other AP2 domain proteins. The amino acid sequence of BNM3A, beginning at position 208, and spanning the first AP2 domain repeat (AP2 domain repeat 1), the second AP2 domain repeat (AP2 domain repeat 2), and the linker region lying between the two repeats (linker), was aligned with the amino acid sequence of other proteins containing two AP2 domains. The amino acid similarity in this region ranges from 53% for APETALA2 to 80% for ZMMHCF1. Identical amino acids are indicated by (*) and gaps are indicated by (-). Protein names are indicated on the left and are abbreviated as follows: ANT, AINTEGUMENTA (accession number U41339); ZM, ZMMHCF1 (accession number Z47554); GL15, GLOSSY15 (accession number U41466); AP2, APETALA2 (accession number U12546).

Figure 6 shows the results of gel blot analysis with a *BNM3A* cDNA fragment performed on RNA extracted from the indicated tissues. RNA gel blots contain either 5 μg (a) or 20 μg (b, c) of total RNA. Figure 6A shows the pattern of *BNM3* expression in microspore embryo cultures. RNA was isolated from late uninucleate microspores and early binucleate pollen at the time of collection (pollen 0d), after four days in culture at 32° C (+ embryo), after four days in culture at 25 °C (pollen 4d), after one day of culture at 25° C, followed by three days of culture at 32 ° C (- embryo) and microspore-derived embryos at the globular, heart, torpedo, 21 day old cotyledon (21 d cot), 28 day old cotyledon (28 d cot) and 42 day old cotyledon (42 d cot) stage of development. *BNM3* expression is detected in embryogenic microspores and developing microspore-derived embryos, but is absent from developing microspores and pollen collected prior to tissue culture and in non-embryogenic samples. The exposure time was seven days. Figure 6B shows that *BNM3* gene expression is detected in developing seeds. Seeds were collected at various days after pollination (DAP). These points in development correspond approximately to the globular (7 d), heart (14 d), torpedo (18 d), early cotyledon (21 d),

mid cotyledon (28 d, 35 d) and late cotyledon (42 d) stages of development. The exposure time was 14 days. Figure 6C shows that *BNM3* gene expression is not detected in non-seed tissues. Roots and leaves were collected from 14 day old greenhouse grown plants. Entire flowers as well as excised anthers and pistils were collected from opened flower buds just prior to anthesis. Small and large buds refer to closed flower buds of less than 5 mm or greater than 5 mm in length, respectively. Siliques were collected 16 days after pollination. The exposure time was 14 days.

Figure 7 shows the phenotype of *Brassica napus* and arabidopsis plants transformed with constructs containing the *BNM3* gene under control of a modified *POLYUBIQUITIN* promoter (B) and double enhanced *35S* promoter containing an AMV translational enhancer (A, C-E). Figure 7A shows embryo structures on the leaf margin of a *Brassica* T1 seedling. Figure 7B shows embryo structures on the petiole of an arabidopsis T2 seedling. Figure 7C shows embryo structures on the cotyledon of an arabidopsis T1 seedling. Figure 7D shows a scanning electron micrograph of the abaxial side of an arabidopsis T1 cotyledon. Note the bipolar nature of the embryos, as well as the emergence of a secondary embryo from the surface of a primary embryo (asterisk). Figure 7E shows a semi-thin section through one of the cotyledons of the T1 seedling shown in (Figure 7C). Note the presence of all the major organs and tissue elements of embryo, as well as the development of new embryos on the flanks of the shoot apical meristems and the cotyledons.

Figure 8 shows the increased regenerative capacity of arabidopsis plants transformed with a construct containing the *BNM3B* gene under control of a modified *POLYUBIQUITIN* promoter. Figure 8A shows wild-type and transgenic leaf and hypocotyl explants on medium containing growth regulators. Figure 8B shows wild-type and transgenic roots on medium containing growth regulators. Figure 8C shows wild-type and transgenic leaf and hypocotyl explants on medium without growth regulators. Figure 8D shows wild-type and transgenic root explants on medium without growth regulators

Figure 9 shows characterization of the *AtBBM* gene; the arabidopsis orthologue of *BNM3*. Figure 9A shows the position of the *AtBBM* sequence and selected restriction endonuclease sites on approximately 8 kb of overlapping *Arabidopsis thaliana* ecotype C24 genomic DNA. The predicted protein coding region of the single arabidopsis *BNM3* homologue spans positions 3426 to 6435 of the genomic sequence. The exons of the predicted coding region are shown as black boxes above the restriction map. A vertical arrow at position 7479 indicates the start of the *IXR3* (*Irregular xylem3*) sequence. The horizontal scale bar is in kilobases. Figure 9 B shows that the arabidopsis *AtBBM* genomic clones and the arabidopsis homologue identified through DNA gel blot analysis using a *Brassica napus BNM3* cDNA probe are the same. Genomic DNA from arabidopsis ecotypes Landsberg *erecta* (L) and Columbia (C) was digested with the restricted enzymes shown in (A) and hybridised to a full-length *BNM3A* cDNA probe (SEQ ID NO1). Comparison of the restriction map shown in (A) with the pattern of hybridising restriction fragments indicates that the full-length *Brassica* cDNA probe detects a single *Arabidopsis* homologue under moderate stringency wash conditions (0.2X SSC, 0.1% SDS at 25 °C). The molecular size marker (in kilobases) is indicated to the right of the blot.

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to asexual embryo formation and regeneration in plants. More specifically, it relates to processes for producing asexually-derived embryos, and for enhancing regeneration capacity in plants. The present invention also relates to heterologous protein production systems in plants, and the uses thereof.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

Genes preferentially expressed during the induction of *Brassica napus* c.v. Topas microspore embryogenesis were isolated via subtractive screening. Seven independent cDNA clones, comprising six unique DNA sequences were found to be differentially expressed between cDNA libraries prepared from embryogenic and non-embryogenic microspore cultures. Several of these *BNM* (for *Brassica napus* microspore embryo) clones, *BNM3A* (SEQ ID NO:1) and *BNM3B* (SEQ ID NO:3), were characterized as described herein. *BNM3A* and *BNM3B* encode the amino acid sequences disclosed in SEQ ID NO:2, and SEQ ID NO:4, respectively. The genomic sequence of *BNM3A* (SEQ ID NO:5), including the regulatory region (nucleotides 1-1619 of SEQ ID NO:5), was also obtained. The arabidopsis orthologue of the *BNM3* gene, called *AtBBM*, was also identified. The genomic sequence of *AtBBM* is depicted in SEQ ID NO:6 while the predicted amino acid sequence is shown in SEQ ID NO:7.

"Regeneration", as used herein, refers to a morphogenetic response that results in the production of new tissues, organs, embryos, whole plants or fragments of whole plants that are derived from a single cell, or a group of cells. Regeneration may proceed indirectly via a callus phase or directly, without an intervening callus phase. "Regenerative capacity" refers to the ability of a plant cell to undergo regeneration.

By "embryogenic cell", it is meant a cell that has completed the transition from either a somatic or a gametophytic cell to a state where no further applied stimuli are necessary to produce an embryo.

By "regulatory element" it is meant those that include developmentally regulated, tissue specific, inducible and constitutive regulatory elements. A regulatory element that is developmentally regulated, or controls the differential expression of a gene under its control, is activated within certain organs or tissues of an organ at specific times during the development of that organ or tissue. However, some regulatory elements that are developmentally regulated may preferentially be active within certain organs or tissues at specific developmental stages, they may also be active in a developmentally regulated manner, or at a basal level in other organs or tissues within the plant as well, such regulatory elements are considered "tissue specific". Regulatory elements may be found either upstream, within, downstream, or a combination thereof, of the coding region of a gene.

An inducible regulatory element is one that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor, that binds specifically to an inducible regulatory element to activate transcription, is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible regulatory element may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods.

A constitutive regulatory element directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive regulatory elements include promoters associated with the CaMV 35S

transcript. (Odell et al., 1985, *Nature*, **313**: 810-812), the rice actin 1 (Zhang et al, 1991, *Plant Cell*, **3**: 1155-1165) and triosephosphate isomerase 1 (Xu et al, 1994, *Plant Physiol.* **106**: 459-467) genes, the maize ubiquitin 1 gene (Cornejo et al, 1993, *Plant Mol. Biol.* **29**: 637-646), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et al, 1995, *Plant Mol. Biol.* **29**: 637-646), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995 *Plant Mol. Biol.* **29**: 995-1004).

By "gene of interest" it is meant any gene that is to be expressed in a transformed plant. Such a gene of interest may include, but is not limited to, a gene that encodes a pharmaceutically active protein, for example growth factors, growth regulators, antibodies, antigens, their derivatives useful for immunization or vaccination and the like. Such proteins include, but are not limited to, interleukins, insulin, G-CSF, GM-CSF, hPG-CSF, M-CSF or combinations thereof, interferons, for example, interferon-α, interferon-ß, interferon-τ, blood clotting factors, for example, Factor VIII, Factor IX, or tPA or combinations thereof. A gene of interest may also encode an industrial enzyme, protein supplement, nutraceutical, or a value-added product for feed, food, or both feed and food use. Examples of such proteins include, but are not limited to proteases, oxidases, phytases chitinases, invertases, lipases, cellulases, xylanases, enzymes involved in oil biosynthesis etc. Other protein supplements, nutraceuticals, or a value-added products include native or modified seed storage proteins and the like.

The present invention is further directed to a chimeric gene construct containing a DNA of interest operatively linked to a regulatory element of the present invention. Any exogenous gene, or gene of interest, can be used and manipulated according to the present invention to result in the expression of the exogenous gene.

The activation of the expression of a gene of interest may also be under the control of a regulatory element that itself is activated by a BNM3 protein. For example, which is not to be considered limiting, a gene of interest may be fused to the napin promoter, and the napin promoter may be induced by BNM3. Furthermore, a gene of interest may be expressed within somatic tissues under the control of one or more

regulatory elements induced by BNM3, so that, as will be described in more detail below, the somatic tissue develops into a seed-like structure comprising embryogenic cells, and these seed-like structures produce the products of the gene of interest.

The chimeric gene construct of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon. Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene of the present construct can therefore be used to construct chimeric genes for expression in plants.

The chimeric gene construct of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the regulatory element selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable

markers include enzymes which provide for resistance to an antibiotic such as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β-glucuronidase), fluorescence, or luminescence, such as luciferase are useful.

Also considered part of this invention are transgenic plants containing a gene or chimeric gene construct of the present invention comprising a *BNM3* gene, a regulatory element obtained from *BNM3*, or the coding region from *BNM3* in operative association with a constitutive, developmental or inducible regulatory element, or a combination thereof. Methods of regenerating whole plants from plant cells are known in the art. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques. The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, biolistics etc. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); Geierson and Corey, *Plant Molecular Biology*, 2d Ed. (1988); and Miki and Iyer, Fundamentals of Gene Transfer in Plants. In *Plant Metabolism*, 2d Ed. DT. Dennis, DH Turpin, DD Lefebrve, DB Layzell (eds), Addison Wesly, Langmans Ltd. London, pp. 561-579 (1997). The present invention further includes a suitable vector comprising the gene or the chimeric gene construct.

A class of genes have been isolated from *Brassica napus* microspore embryo cultures. These genes have been found to be important regulators of embryogenesis by their ability to induce the formation of asexually-derived embryos when ectopically expressed in the vegetative tissues of plants. These genes are hereinafter indicated as

BNM3 genes (*Brassica napus* microspore embryo). SEQ ID NO. 1 depicts the cDNA of *BNM3A* , SEQ ID NO. 3 depicts the cDNA of *BNM3B,* and the genomic sequence for *BNM3A* is given in SEQ ID NO:5. The regulatory region of BNM3A lies within nucleotides 1-1619 of SEQ ID NO:5. The predicted protein sequences encoded by the DNAs of SEQ ID NO. 1 and 3 are outlined in SEQ ID NOs. 2 and 4, respectively.

The orthologue of the BNM3 gene has also been identified in arabidopsis and is hereinafter referred to as *AtBBM.* SEQ ID NO:6 depicts the genomic sequence for *AtBBM.* The regulatory region of *AtBBM* lies within nucleotides 1-2025 of SEQ ID NO:6. The predicted amino acid sequence is given in SEQ ID NO:7.

The *BNM3* translation products contain two copies of an AP2 domain separated by a linker region (Figure 3; amino acids 208-378 of SEQ ID NO: 2, and SEQ ID NO: 4, nucleotides 2694-4252 of SEQ ID NO:5, nucleotides 2938-4416 of SEQ ID NO:6), which herein is referred to as "AP2domain" or "AP2 domain repeat1-linker-AP2domain repeat2". The AP2 domain is thought to mediate protein-protein interactions. The ability of a number of AP2 domain containing proteins to bind DNA, coupled with the presence of putative nuclear localization signals and acidic regions that may function as transcriptional activators suggests these proteins function as transcription factors. The AP2 domain repeat1-linker-AP2domain repeat2 of BNM3 exhibits about 99% homology with the AP2 domain of AtBBM (95% nucleotide similarity between *BNM3* and *AtBBM*), as well as a high degree of similarity with other AP2-comprising proteins, for example, ANT about 85% (76% nucleotide similarity), MOE17 (chromosome 3) about 85% (78% nucleotide similarity; if the introns are included in the comparison, for example the AP2 region from SEQ ID NO5, then MOE17 exhibits about 63% nucleotide similarity over a 285 bp region within the second AP2 domain), ZMMHCF1, 88%, or GLOSSY15, 66%. However, outside the two AP2 domains, the similarity of the sequence of BNM3 and these other AP2-containing proteins decreases significantly.

By *"BNM3"* or "*BNM*3 gene", it is meant the sequence of oligonucleotides as disclosed in SEQ ID NOs:1, 3, 5, or 6, or fragments, derivatives, or mutations thereof, or oligonucleotide sequences that exhibit at least:

i) 70% homology or similarity, with a fragment or derivative of the sequences disclosed in SEQ ID NOs 1, 3, 5 or 6, not including the AP2 domain repeat1-linker-AP2 domain repeat2 region as defined by nucleotides 741-1257 of SEQ ID NO;1 nucleotides 672-1188 of SEQ ID NO:3, the corresponding sequence within coding region of nucleotides 2694-4252 of SEQ ID NO:5, or nucleotides 2938-4416 of SEQ ID NO:6 (the regions defined by nucleotides 2694-4252 of SEQ ID NO:5 or nucleotides 2938-4416 of SEQ ID NO:6, are interrupted by 7 introns; see Figure 9); or

ii) 70% homology or similarity, with the full length of sequences disclosed in SEQ ID NOs 1, 3, 5 or 6 including the AP2 domain repeat1-linker-AP2 domain repeat2 region.

Such homology determinations may be made using oligonucleotide alignment algorithms for example, but not limited to a BLAST (GenBank URL: www.ncbi.nlm.nih.gov/cgi-bin/BLAST/, using default parameters: Program: blastn; Database: nr; Expect 10; filter: default; Alignment: pairwise; Query genetic Codes: Standard(1)) or FASTA, again using default parameters. Using sequence similarity searches *AtBBM* exhibits about 85% homology with the full length of *BNM3*, and therefore, *AtBBM* is a BNM3 gene. Furthermore, a *BNM3* gene may also be defined in terms of its ability to hybridize with sequences disclosed in the present invention. Therefore, *"BNM3"* or "*BNM*3 gene", also includes:

iii) oligonucleotides from greater than about 15 nucleotides in length, preferably 20 to 25 nucleotides in length, that associate with any of SEQ ID NO:1, 3, 5 or 6, or a fragment or derivative of the sequences disclosed in SEQ ID NOs 1, 3, 5 or 6, not including the region coding for the AP2 domain (AP2 domain repeat1-

linker-AP2 domain repeat 2) as defined above, under conditions of high stringency, for example, but not to be limited to, hybridization using gel blots (Southern hybridization) at about 65°C at 5X SSC, followed by wash conditions at 0.1X SSC, at 65°C; or

iv) substantially full length nucleotide sequences, or nucleotide sequences of greater than about 1000 nucleotides in length, that associate with SEQ ID NO:1, or 3, or a fragment or derivative of the sequences disclosed in SEQ ID NOs 1, 3 of a length greater than about 1000 nucleotides, not including the region coding for the AP2 domain (AP2 domain repeat1-linker-AP2 domain repeat 2) as defined above, under conditions of moderate or high stringency, for example, but not to be limited to, hybridization using gel blots (Southern hybridization) at about 25°C at 0.2XSSC, 0.1% SDS, or 65°C 5XSSC, respectively, followed by wash conditions at 0.2XSSC, 0.1% SDS at 25°C.

Under conditions of moderate stringency, the full length BNM3 cDNA only hybridizes with fragments of *AtBBM* (see Figure 9B), and therefore, *AtBBM* is a BNM3 gene. Sequence analysis of the *AtBBM* genomic clones position the 5' end of the *IRREGULAR XYLEM3* (*IXR3*) gene downstream of the putative *AtBBM* coding region (position 7479, Figure 9A). *IXR3* has previously been shown to map to a 150 kb region of chromosome 5 between the markers nga106 (33.26 cM) and mi438 (33.34 cM) (Taylor et al., 1999). This data indicates that the arabidopsis orthologue of the *Brassica BNM3* genes is encoded by a single gene that maps to chromosome 5. A sequence highly similar to *AtBBM*, TAMU BAC clone:T10B6 (accession number AP002073; Nakamura, May 18, 2000) has also been mapped to chromosome 5.

"*BNM*3 gene" also includes DNA molecules that comprises at least 27 contiguous nucleotides of SEQ ID NOs:1, 3, 5, or 6 or at least 22 contiguous nucleotides within the regulatory region of nucleotides 1-1619 of SEQ ID NO:5, or nucleotides 1-2025 of SEQ ID NO:6. A fragment of *BNM*3, as defined may be used as a probe for the identification of nucleotides related to *BNM*3 regulatory, or coding, regions within an organism, or as

primers for the amplification of these nucleotide sequences. Furthermore, molecules comprising at least 27 contiguous nucleotides, and preferably greater than about 30 to about 35 nucleotides, of the sequence of SEQ ID NOs:1, 3, 5 or 6 and that encode a protein, or an active fragment thereof, that when present at a sufficient level within a plant cell renders the cell embryogenic, increases the regenerative capacity of the plant cell, or renders the cell embryogenic and increases the regenerative capacity of the plant cell, are also considered to be *BNM3* genes. Preferably, a *BNM3* gene comprises from about 50 to about 1981 nucleotides of SEQ ID NOs: 1 or 3, from about 50 to about 3538 nucleotides from the coding region (1620-4858) of SEQ ID NO:5, or from about 50 to about 3009 nucleotides from the coding region (2025-5035) of SEQ ID NO: 6.

The genomic *BNM3* sequences obtained from *Brassica napus* and arabidopsis are characterized as comprising 8 introns. These introns are found at nucleotides 1846-2298, 2720-2952, 3036-3160, 3170-3314, 3404-3553, 3628-3797, 3849-3961, and 4039-4148, of SEQ ID NO:5, and nucleotides 2249-2578, 2994-3220, 3304-3420, 3429-3521, 3611-3770, 3845-3969, 4020-4151 and 4229-4310 of SEQ ID NO:6. The start codon of SEQ ID NO's:5 and 6 are at nucelotides 1620 and 2026, respectively, while the stop codons are found at positions 4856 and 5035, respectively.

By "*BNM3* regulatory region" it is meant the sequence of oligonucleotides that exhibit the property of regulating the expression of (either positively, for example an enhancer or promoter region, or negatively, for example a silencer region), and that are in operative association with, a *BNM3* gene. Typically the *BNM3* regulatory region comprises nucleotides upstream from the start site of a *BNM3* gene, however, sequences residing within other regions of the gene may also exhibit regulatory properties and be considered a *BNM3* regulatory region, for example but not limited to sequences within introns. An example of a *BNM3* regulatory region, which is not to be considered limiting, includes:

- a sequence operably linked with a *BNM3* gene that exhibits a regulatory function, for example but not limited to a regulatory region upstream from the start site of a BNM3 gene or within an intron, or a fragment, derivative, or mutation thereof;
- nucleotides from about 1 to about1619 in SEQ ID NO:5 or a fragment or derivative thereof;
- nucleotides from about 1 to about 2025 of SEQ ID NO:6 or a fragment or derivative thereof;
- a nucleotide sequence that associates with a nucleotide sequence from about 1000 to about1619 of SEQ ID NO:5 or from about 1500 to about 2025 of SEQ ID NO:6, or a fragment or derivative thereof, under conditions of high stringency, for example, but not to be limited to, hybridization to gel blots at about 65°C in 5XSSC, followed by wash conditions at 0.1X SSC, 65°C;
- a nucleotide sequence that associates with a nucleotide sequence from about 1 to about1000 of SEQ ID NO:5 or from about 1 to about 1500 of SEQ ID NO:6, or a fragment or derivative thereof, under conditions of moderate or high stringency, for example, but not to be limited to, hybridization to gel blots at about 25°C in 0.2XSSC, 0.1% SDS, or 65°C in 5XSSC, respectively, followed by wash conditions in 0.1X SSC, at 65°C; or
- a nucleotide sequence that exhibits at least 70% similarity with the nucleotide sequence from about 1000 to about1619 of SEQ ID NO:5 or from about 1500 to about 2025 of SEQ ID NO:6, or a fragment thereof of at least about 22 nucleotides, as determined using oligonucleotide alignment (for example, but not limited to a BLAST or FASTA search, using default parameters; see above).

By "BNM3 protein" it is meant a protein, or a biologically active fragment thereof, that renders a plant cell embryogenic, increases the regenerative capacity of the plant cell, or renders the cell embryogenic, increases the regenerative capacity of the plant cell, and that is encoded by a *BNM3* gene, as defined above. Preferably, a BNM3 protein comprises from about 30 to about 579 amino acids of the sequence disclosed in SEQ ID NO:2, from about 30 to about 579 amino acids of the sequence disclosed in SEQ

ID NO: 4, or from about 30 to about 581 amino acids of the sequence disclosed in SEQ ID NO:7. However, BNM3 protein may also be defined as a protein having at least 70% homology with either SEQ ID NO:2, 4, or 7 not including the AP2-repeat1-linker-AP2 repeat2 region (amino acids 208-378 of SEQ ID NO:s 2, and 3, amino acids 205-375 of SEQ ID NO:7).

Search of the sequence databases indicated that the *BNM3* translation products contain two copies of an AP2 domain (Figure 3; see also SEQ ID NO: 2 for BNM3A, SEQ ID NO: 4 for BNM3B, and SEQ ID NO:7 for AtBBM). The AP2 domain was first identified in APETALA2, an arabidopsis protein that regulates meristem identity, floral organ specification, seedcoat development and floral homeotic gene expression (Jofuku *et al*., 1994), but has since been identified in a wide range of proteins with diverse functions.

The AP2 domain is usually between 58 to 68 amino acids in length and contains a conserved central core of 18 amino acids, characterized by its ability to form an amphipathic α helix, a structure thought to mediate protein-protein interactions. The ability of a number of AP2 domain containing proteins to bind DNA, coupled with the presence of putative nuclear localization signals and acidic regions that may function as transcriptional activators suggests these proteins function as transcription factors.

Two phylogenetically distinct classes of AP2 domain proteins have been identified; proteins with a single AP2 domain (EREBP-like) and proteins with two AP2 domains (AP2-like; (Zhou, 1997)). The proteins encoded by the genes of this invention represent unique members of the latter class of proteins.

Accordingly, an aspect of the present invention provides for an isolated DNA molecule that comprises a sequence encoding a protein that contains two AP2 domains. The protein, when present at a sufficient level in a plant cell, renders the cell embryogenic, increases the regenerative capacity of the cell, or both renders the cell embryogenic and increases the regenerative capacity of the cell.

Analysis of *BNM3* expression during microspore-derived embryo development, seed development, or non-seed tissue development, using Northerns (Figure 6) indicated that the *BNM3* genes are preferentially expressed in embryogenic microspore cultures, microspore-derived embryos and seeds. *BNM3* transcripts were not detected in any of the non-seed tissues tested.

BNM3 mRNA is detected in microspore cultures induced to undergo embryogenesis, as well as in the subsequent globular, heart, torpedo and cotyledon stages of microspore-derived embryo development (e.g. Figure 6A). RNAs are also detected within developing seeds, 14 days after pollination (14 DAP), corresponding to the heart stage of embryo development. *BNM3* expression increases during the early (21 DAP) and mid-cotyledon (28 DAP) stages of embryo development and remains constant thereafter (Figure 6B).

Constitutive expression of *BNM3* resulted in the formation of somatic embryos on vegetative structures such as cotyledons, petioles, leaf blades and the shoot apical meristem of plants (Figure 7). In these experiments *BNM3* cDNAs were placed under the control of two separate constitutive promoter constructs, a modified sunflower *POLYUBIQUITIN* promoter construct, and a double enhanced *35S* promoter construct containing an AMV translational enhancer, however, it is to be understood that any suitable constitutive promoter may be used for this purpose. Such *BNM3*-derived ectopic embryos contain all of the organ systems and tissue layers found in the developing zygotic embryo in that these embryos are bipolar (Figure 7E), consist of an axis, a hypocotyl and radicle region, shoot and root meristems, and cotyledons. In addition, each organ system contained the characteristic radial arrangement of three specialized tissue layers (epidermis, ground parenchyma and provascular tissue) found in zygotic embryos. Continued expression of the *BNM3* gene within the developing ectopic embryo leads to a reiteration of the embryo-forming process, with the result that new embryos are continuously formed on the surface of pre-existing embryos (Figure 7E).

Constitutive expression of *BNM3* results in the increased ability of a plant to regenerate shoots *in vitro* in the presence of added growth regulators. Root explants from transgenic plants ectopically expressing *BNM3* show at least a 5-fold increase in shoot regeneration in the presence of hormones as compared to root explants obtained from wild-type plants (Figure 8A,B). Shoots also developed faster in the transgenic explants, compared to the wild-type. Wild-type leaf and hypocotyl explants initially responded by producing callus on the cut end of the petiole (Figure 8B) followed by callus formation along the length of the petiole. In contrast, explants from transgenic lines immediately produced new shoots (Figure 8B) or roots from the cut end of the petiole. Explants that initially produced roots eventually also produced shoots.

Transgenic explants, constitutively expressing *BNM3* were also able to regenerate in the absence of added growth regulators. These explants, when placed on media lacking growth regulators regenerated shoots either from the cut end of the leaf and hypocotyl explants or from the nodule-like structures of root explants (Figures 8C,D). In all cases regenerated shoots developed, rooted, flowered and set seed. Conversely, wild-type leaf and hypocotyl explants placed on medium lacking growth regulators occasionally produce callus or roots at the cut end of the leaf petiole, however no shoots form from these structures (Figure 8C,D).

It is also considered within the scope of the present invention, that expression of *BNM*3 may be used to initiate a developmental cascade within a transformed plant or plant cell. This cascade may arise as a result of the stable integration of a DNA-based vector expressing BNM3 within a transformed plant, however, such a cascade may also arise as a result of transient expression of *BNM*3, and does not require the stable integration of the *BNM*3-based vector within a plant cell. These transient approaches may be useful for inducing somatic embryogenesis, gametophytically-derived embryogenesis, or increasing the regenerative capacity of a plant or plant cell.

Plants in which a *BNM3* gene is ectopically expressed exhibit advantageous qualities including:

- formation of asexually derived embryos;
- increased regenerative capacity of tissue explants;
- the ability of tissue explants to regenerate in the absence of added plant growth regulators; and
- the expression of seed components in non-seed organs in which *BNM3* is ectopically expressed.

Furthermore, plants that ectopically express at least one BNM3 gene can be used for the production of recombinant proteins using seed specific regulatory elements.

For the applications of BNM3 as described below, it will be advantageous to obtain a high level of the *BNM3* transcript and/or BNM3 protein in order to obtain plants in which the phenotype is highly penetrant. This may be obtained by using genetic elements such as introns, transcriptional enhancers or translational enhancers which are known to enhance gene or protein expression levels.

The BNM3 sequences of the present invention may be used for several applications including, but not limited to, the control of embryo processes, the control of regeneration processes, the use of regulatory sequences for targeted gene expression, the use of BNM3 sequences as selectable markers of transformed plants, or for embryogenic cells. These applications are disclosed in more detail below.

<u>Use of *BNM3* Sequences to Control Embryogenic Processes</u>

As described herein, *BNM3* genes play an important role in initiation and maintenance of embryo development. *BNM3* genes have been found in a wide range of members of the plant kingdom. Regulatory regions obtained from these genes may be used to control the transcription of *BNM3* or a derivative or fragment thereof, or any gene of interest, using methods known to one of skill in the art.

Ectopic expression of a *BNM3* gene is sufficient to induce recurrent formation of asexually derived embryos on the vegetative tissues of plants (see example 4). Depending upon the promoter used, ectopic over-expression of *BNM3* genes may be used to produce somatic or gametophytic embryos. Somatic or gametophytic embryos may be obtained by expressing a *BNM3* gene under the control of a constitutive regulatory element, as is shown in Example 5, or may also be obtained by expressing a *BNM3* gene under the control of tissue specific or developmentally regulated elements, inducible elements derived from either plant or non-plant genes or through transient expression. In this respect, chemical induction systems (e.g. see Gatz and Lenk, 1998, which is incorporated by reference) or transient expression using methods which do not result in stable integration of the *BNM3* gene, or which make direct use of the BNM3 protein e.g. microprojectile bombardment of DNA or protein may also be employed.

Temporal and/or spatial restriction of *BNM3* expression using inducible, tissue specific or developmentally regulated elements, is preferred when recurrent embryogenesis is not a desirable trait. The regulatory elements used to restrict *BNM3* to a specific developmental stage or cell type will depend on the application. For example, regulatory elements that may be used to express *BNM3* for the production of microspore-derived embryos include, but are not limited to, those of the class I low molecular weight heat shock inducible gene, *GMHSP17.3B* (Zarsky *et al*., 1995, which is incorporated by reference), or microspore/pollen expressed genes such as *NTM19* (Custers *et al*., 1997, EP 790,311, which are incorporated by reference), *BCP1* (Xu *et al*., 1995, which is incorporated by reference), *LAT52* (Twell *et al*., 1989, which is incorporated by reference), *BNM*1 (Treacy et al 1997, which is incorporated by reference) and *APG* (Roberts *et al*., 1993, which is incorporated by reference).

Examples of regulatory elements that may be used to express *BNM3* for the production of somatic embryos include, but are not limited to, those of genes activated by plant growth regulators which are routinely used to induce somatic embryogenesis in tissue culture. Specific examples, which are to be considered non-limiting, include the cytokinin inducible *IB6* and *CKI1* genes (Brandstatter and Kieber, 1998; Kakimoto,

1996, which are incorporated by reference) and the auxin inducible element, DR5 (Ulmasov *et al.*, 1997, which is incorporated by reference). However, it is to be understood that other regulatory elements may be included for the expression of *BNM3* in plants.

Furthermore, examples of gene regulatory elements suitable for directing expression of *BNM3* to obtain adventitious embryony include, but are not limited to, those obtained from the ovule and embryo expressed *SERK* gene (Schmidt et al, 1997 which is incorporated by reference), the ovule expressed *AGL11* gene (Roundsley *et al.*, 1995, which is incorporated by reference), the nucellus expressed *NUC1* gene (Doan *et al.*, 1996; WO 98/08961, which are incorporated by reference), or the inner integument-expressed genes, *FBP7* (Angenent *et al*, 1995, which is incorporated by reference) and *SC4* (US application 09/059,909, filed April 13, 1998, which is incorporated by reference) genes.

According to one aspect of the present invention there is provided a method for the efficient production of microspore-derived embryos in plants. This method involves:

i) transforming a plant of interest, for example, *Brassica napus* (using transformation techniques known to one of skill, for example, DeBlock *et al.*, 1989, Clough and Bent 1998, Vergunst et al. 1998, Klein et al 1987, which are incorporated herein by reference) with a vector construct, or isolated DNA, consisting of a *BNM3* gene under control of a suitable regulatory element, which may be constitutive, tissue specific, developmentally regulated, or inducible and, optionally, a marker gene for selection of transformants;
ii) selecting transformed plants;
iii) producing lines that ectopically overexpress the *BNM3* gene, or BNM3 protein;
iv) isolating microspores and pollen from the transgenic lines and culturing microspores and pollen to induce embryogenesis.

Embryogenesis can be induced by any suitable protocol, for example, which is not to be considered limiting, culturing microspore and pollen for about four days at from about 28° to about 35 °C, preferably at about 32 °C, then transferring embryogenic cells or embryos to about 25 °C.
Using the above method, *Brassica napus* cultivars ectopically overexpressing *BNM3* show an increase in the percentage of embryogenic cells or embryos over that observed when microspores or pollen are prepared from wild-type plants that do not ectopically express *BNM3*.

Examples of regulatory elements that may be used to express *BNM3* for the production of microspore-derived embryos include, but are not limited to, those of the class I low molecular weight heat shock inducible gene, *GMHSP17.3B* (Zarsky *et al.*, 1995, which is incorporated by reference), or microspore/pollen expressed genes such as *NTM19* (Oldenhof *et al.*, 1996, EP 790,311, which are incorporated by reference), *BCP1* (Xu *et al.*, 1995, which is incorporated by reference), *LAT52* (Twell *et al.*, 1989, which is incorporated by reference), *BNM*1 (Treacy et al 1997, which is incorporated by reference), and *APG* (Roberts *et al.*, 1993, which is incorporated by reference). Also useful are inducible regulatory elements, for example but not limited to, tetracycline-inducible promoter (Gatz 1997, which is incorporated by reference), steroid inducible promoter (Aoyama and Chua 1997, which is incorporated by reference) and ethanol-inducible promoter (Slater et al 1998, Caddick et al. 1998, which are incorporated by reference).

In a similar fashion, microspore-derived embryos may also be produced in plants by introducing into a plant of interest a BNM3 protein, (e.g. via biolistics ; Klein et al 1987) and selecting for plants that exhibit increased microspore embryogenesis.

This invention also provides a method for the efficient production of somatic embryos *in vitro*. This method involves:

i) transforming a plant, for example, arabidopsis using transformation techniques known to one of skill (for example, but not limited to, DeBlock *et al.*, 1989, Clough and Bent 1998, Vergunst et al. 1998, which are incorporated by reference), or a plant cell may also be transiently transformed using methods known to one of skill (for example, biolistics; Klein et al 1987) with a vector construct containing a *BNM3* gene under control of suitable regulatory element, which may be constitutive, inducible or developmentally regulated, and, optionally, a marker gene for selection of transformants is transformed to several arabidopsis.

ii) selecting transformed plants, and

iii) culturing the desired explant from the selected transformed plants, for example, but not limited to, root, leaf or seedlings *in vitro*, in media with or without appropriate growth regulators, for example, but not limited to 2,4-D (e.g. Mordhorst *et al.*, 1998) to produce direct embryogenesis or embryogenic callus; and

iv) transferring embryos, non-embryogenic callus, or both embryos and non-embryogenic callus to appropriate media for the production of embryos, plantlets, or both embryos or plantlets.

For example, when the results of the above method are compared with the production of somatic embryos *in vitro* using a number of arabidopsis ecotypes, directed embryogenesis or embryogenic callus is initiated at a higher frequency from transgenic lines ectopically over-expressing *BNM3* than in wild-type controls.

Examples of regulatory elements that may be used to express *BNM3* for the production of somatic embryos include, but are not limited to, those of genes activated by plant growth regulators which are routinely used to induce somatic embryogenesis in tissue culture. Specific examples, which are to be considered non-limiting, cytokinin inducible *IB6* and *CKI1* genes (Brandstatter and Kieber, 1998; Kakimoto, 1996, which are incorporated by reference) and the auxin inducible element, DR5 (Ulmasov *et al.*, 1997, which is incorporated by reference). Also useful are inducible regulatory elements,

for example but not limited to, a tetracycline-inducible promoter (Gatz 1997, which is incorporated by reference), a steroid inducible promoter (Aoyama and Chua 1997, which is incorporated by reference), and an ethanol-inducible promoter (Slater et al 1998, Caddick et al. 1998, which are incorporated by reference).

Ectopic initiation of embryo development is one of the key steps in apomixis. As shown in Example 4, ectopic expression of a *BNM3* gene is sufficient to initiate embryo formation in otherwise non-embryo-forming tissue. A *BNM3* gene may therefore be used to initiate adventitious embryony or parthenogenesis of a reduced or unreduced embryo sac cell by expression of the gene in the sporophytic or gametophytic tissues of the developing ovule.

Adventitious embryony is achieved by expressing *BNM3* in sporophytic ovule tissues such as the nucellus, the inner integuments or other tissues lying adjacent to or in proximity to the developing embryo sac. This method involves:

i) transforming a desired plant (see above methods) with a vector construct consisting of a *BNM3* gene under control of suitable regulatory element, which may be constitutive, inducible or developmentally regulated, and, optionally, a marker gene for selection of transformants, using methods known within the art;
ii) selecting transformed plants;
iii) emasculating the transformed plant;
iv) pollinating the transformed plants with pollen carrying one or more dominant selectable markers, for example GUS or kanamycin resistance; and
v) assaying for production of clonal offspring.

When the results of the above method are compared with the pollination of a wild-type arabidopsis plant with pollen carrying the dominant selectable marker, all F1 embryos resulting from this cross inherit the dominant marker while embryos derived

from plants ectopically over expressing the BNM3 gene or protein are clonally derived via sexual embryo formation and do not inherit the dominant selectable marker.

Specific examples of gene regulatory elements suitable for directing expression of *BNM3* to obtain adventitious embryony, diplospory or haploid parthenogenesis of embryo sac components include the ovule expressed SERK gene (Schmidt et al. 1997, which is incorporated by reference), the meiosis expressed *AtDMC1* gene, (Klimyuk and Jones, 1997; WO 98/28431, which are incorporated by reference), the ovule expressed *AGL11* gene (Roundsley *et al.*, 1995, which is incorporated by reference), the nucellus expressed *NUC1* gene (Doan *et al.*, 1996; WO 98/08961, which are incorporated by reference), and the inner integument-expressed genes, *FBP7* (Angenent *et al*, 1995, which is incorporated by reference) and *SC4* (US application 09/059,909, filed April 13, 1998, which is incorporated by reference) genes. Furthermore, inducible systems, for example but not limited to, tetracycline-inducible promoter (Gatz 1997, which is incorporated by reference), steroid inducible promoter (Aoyama and Chua 1997, which is incorporated by reference), ethanol-inducible promoter (Slater et al 1998, Caddick et al. 1998, which are incorporated by reference) may also be used. Parthenogenesis from cells of the embryo sac requires a regulatory element that is active in one or more cells of the female gametophyte or their precursors. Fertilization of the meiotically-derived polar nuclei is desirable when the development of seed is dependent on the presence of endosperm.

<u>Use of *BNM3* Sequences to Control Regeneration Processes</u>

Plants ectopically over-expressing the *BNM3* genes exhibit increased regenerative capacity and the ability to regenerate whole plants in the absence of added growth regulators (see example 5). *BNM3* gene expression may therefore be used to enhance or induce the regeneration capacity of plant tissues *in vivo* or *in vitro*. The regulatory elements used to express *BNM3* will depend, in part, on the target tissue used for regeneration. Regeneration of plant tissues may be obtained by expressing a *BNM3* gene under the control of a constitutive regulatory element, for

example, but not limited to, 35S, or by expressing a *BNM3* gene under the control of tissue specific or developmentally regulated elements, inducible elements derived from either plant or non-plant genes (e.g. Gatz and Lenk, 1998, which is incorporated by reference), or through transient expression methods which do not result in stable integration of the *BNM3* gene or which make direct use of the BNM3 protein (e.g. microprojectile bombardment of DNA or protein). Chemical induction systems (see Gatz and Lenk, 1998) or regulatory elements of genes that respond to plant growth regulators used to induce regeneration, such as, for example, cytokinin (Brandstatter and Kieber, 1998; Kakimoto, 1996) or auxin (Ulmasov et al., 1997), or genes expressed at the wound site of tissue explants (Xu *et al.*, 1993) may be used.

A further application is the use of a *BNM3* gene as a selectable marker for the recovery of transgenic plants. As an example of this application which is not to be considered limiting in any manner, roots of a seedling, for example, an *Arabidopsis* ecotype C24 seedling, are cocultivated with a single *Agrobacterium tumefaciens* strain (per Vergunst *et al*, 1998; except that all steps are carried out in the absence of added growth regulators) containing two binary constructs:

- a first binary vector carries a reporter gene fusion, for example, but not limited to, *35S:GUS*;
- a second binary vector contains a *BNM3* gene under control of suitable regulatory element.

BNM3 gene expression is activated upon integration of the above construct into the arabidopsis genome and transgenic plants are selected on the basis of their ability to regenerate under conditions in which wild-type explants are unable to regenerate, for example, but not limited to, the absence of growth regulators. In many instances the T-DNA carrying the *BNM3* gene and the T-DNA carrying the gene of interest will integrate at unlinked loci. The T-DNA containing the introduced *BNM3* sequence, and it's associated increased regenerative capacity phenotype, may therefore be removed in the progeny plants by simple segregation (Daley et al. 1998). However, as will be apparent to one of skill in the art, other methods such as transient

expression, which do not result in stable integration of the BNM3 gene or which make direct use of the BNM3 protein, may also be employed.

Use of *BNM3* Sequences to Target Gene Expression to the Embryo

Since *BNM3* genes are preferentially expressed in developing embryos (see example 3), a further application of this invention is the use of *BNM3* regulatory regions to target expression of at least one heterologous gene of interest to the developing embryo for any purpose, for example, but not limited to, altering embryo and seed traits such as seed viability or size, composition of constituents of the seed, disease resistance, or the production of high value products such as vaccines antibodies, biopharmaceuticals or other specialty chemicals.

Use of *BNM3* Expression as a Marker for Embryogenic Cells

As shown in Examples 3 and 4, *BNM3* gene expression is detected during the earliest phase of plant embryogenesis and is itself sufficient to activate signal transduction cascades leading to embryo development. *BNM3* gene expression is therefore a specific marker for the entry of a plant cell into the embryogenic pathway.

BNM3 expression is associated with embryo-forming cell divisions *in vitro* and *in vivo* and as such can be used to define culture conditions that alter the embryo-forming capacity of a tissue *in vitro*. Cells with embryogenic capacity or cells that undergo only a limited number of embryo-forming divisions are difficult to identify in the absence of structures that morphologically resemble embryos. However, these cells may be identified on the basis of *BNM3* expression. In this application, a vector containing the *BNM3* regulatory region, fused to a reporter gene, for example, but limited to, *GUS* (Jefferson *et al.*, 1987), *Luciferase* (Ow *et al.*, 1987) or *GFP* (Haselhoff and Amos, 1995) is transformed to a plant of interest. Homozygous transgenic lines exhibiting high levels of reporter gene expression in the embryo are cultured under *in vitro* conditions. Embryogenic cells, as well as culture conditions

which facilitate or enhance the formation of embryogenic cells are identified on the basis of reporter gene expression within the cultured tissue.

A related application is the use of the *BNM3* gene as a marker in apomictic species for the identification of individual cells that are in the process of forming asexually-derived embryos. In this application, cells entering the autonomous embryo pathway are identified by mRNA *in situ* hybridization using a RNA probe derived from a *BNM3* gene sequence, by immunocytochemistry using a antibody directed against a BNM3 protein, by transforming plants with a DNA construct containing a gene fusion between *BNM3* regulatory regions and a reporter gene, or by any similar technique known to those skilled in the art.

Identification of Signal Transduction Components

Signal transduction components which activate or are activated by *BNM3* gene expression can be elucidated by identifying proteins and DNA sequences that interact with a *BNM3* gene and its protein product. These signal transduction components may be identified using techniques known to a person skilled in the art, including for example, but not limited to:

- mutagenesis to identify intr- and/or extragenic suppressors or enhancers of the *BNM3* gain-of-function phenotype;
- yeast one hybrid screens for the isolation of proteins that bind to the *BNM3* regulatory regions to influence *BNM3* gene expression;
- genetic selection in yeast to identify genes that are direct targets of BNM3 binding;
- DNA arrays or proteomics to identify genes which are activated in a BNM3 signal transduction cascade; and
- yeast two hybrid screens to identify proteins that interact with BNM3 to influence expression of downstream target genes.

Techniques for the analysis of the signal transduction components and signalling components are well known (see for example, Meijer *et al.* (1998), Lipshutz *et al.* (1999), and Anderson and Anderson (1998)).

Plants over-expressing the *BNM3* gene under control of a strong constitutive regulatory element such as, for example, but not limited to, the Cauliflower Mosaic Virus *35S* promoter exhibit ectopic embryo formation, enhanced regeneration via organogenesis or a combination thereof (Examples 4 and 5). The ability of *BNM3* ectopic over-expression to induce both embryo formation and enhance regeneration processes can be used to identify mutants altered in their embryo-forming or regenerative capacity. In this application a vector construct consisting of a *BNM3* protein coding region under control of a regulatory element that is sufficient to promote either ectopic embryo formation or enhanced regeneration phenotype is made and introduced into a plant of interest. Homozygous transgenic lines exhibiting a high penetrance of ectopic embryo formation, enhanced regeneration phenotype, or a combination thereof are identified. These lines are mutagenized by any available technique well known to the person skilled in the art, but which may include EMS mutagenesis, fast neutron mutagenesis, transposon mutagenesis or T-DNA mutagenesis. Mutagenized plants are then screened for alterations in the ectopic embryo formation or regeneration phenotype. These alterations include, for example, but not limited to, elimination or enhancement of the ability to promote ectopic asexual embryo formation or to regenerate in the absence of added growth regulators.

Heterologous Protein Expression System

Genetic control of the signal transduction pathway leading to embryogenesis and organogenesis in non-seed organs of transgenic plants may be activated by ectopic expression of a *BNM*3 gene. Expression of a *BNM*3 gene in association with a heterologous promoter can be used to produce altered seed components including for example, proteins, oils and other metabolites. Biotransformation of desired organs

may also include altering the nutritive value of, for example leaves of forage crops, or it may be used to create alternative uses for crops. The use of promoters that are induced by the signal transduction cascade initiated by expression of *BNM3* can be used to express high-valued recombinant proteins in organs other than seeds. An example of one such promoter is the napin promoter, obtained from the 2S seed storage protein napin. The production of proteins initiated from a *BNM3*-induced cascade, may be achieved within organs exhibiting greater biomass than seeds. Therefore, this technology may be used to create alternatives for plants as crops.

Accordingly, the present invention further relates to a binary system in which the BNM3 protein binds directly or indirectly to an embryo-expressed regulatory sequence (target sequence) and activate transcription of a chimeric gene construct in any plant cell, tissue or organ. Therefore, BNM3 may be used to directly or indirectly activate transcription of a chimeric gene construct. This approach involves BNM3 interacting either directly with at least one target sequence from an embryo-expressed gene, or indirectly by initiating an embryogenic signal cascade that activates a transcription factor that in turn binds to and activates transcription from at least one target sequence. This binary system may be used for the expression of proteins in somatic tissues with the properties of expression in seeds.

In this application transgenic plants containing the *BNM3* gene under control of a constitutive regulatory element, for example, but not limited to the *35S* promoter (*35S:BNM3*) are created to produce a BNM3 activator line. *BNM3* expression may be demonstrated in a wide range of tissues in the BNM3 activator lines by RNA gel blot analysis. Stable homozygous activator lines with high levels of *BNM3* expression are identified. Somatic tissues over-expressing *BNM3* may be examined for expression of other embryo-expressed genes, such as arabin (Guerche et al., 1990), cruciferin (Pang *et al*., 1988) or oleosin, or for morphological properties that are normally characteristic of seeds, such as the presence of lipid or protein bodies.

Transgenic plants of the same species to that used to generate the BNM3 activator lines described above are also created which contain an embryo-expressed promoter fused to a gene of interest, to produce a gene of interest line. In order to help describe this embodiment, the gene of interest line expresses a reporter gene, such as *GUS* , and examples, which are not to be considered limiting, of such lines include *Brassica napus* 2S albumin seed storage protein gene, *BngNAP1:GUS* fusion (Baszczynski *et al.*, 1994) or a *SERK:GUS* fusion (Schmidt *et al.*, 1997; a non-seed expressed reporter construct such as *BNM1:GUS* (Treacy *et al.*, 1997) may be used as a negative control). The fidelity of expression of the gene of interest in the specific organs and tissues of these gene of interest lines is demonstrated for each construct. Stable homozygous lines with high levels of expression of the gene of interest expression are created.

Transgenic lines containing BNM3 activator lines and gene of interest lines are crossed and the progeny seeds collected. *BNM3* gene expression, and in this example, GUS activity, expression of other embryo-expressed genes, as well as the morphological characteristics of transformed tissues, are examined. *BNM3* expression in non-seed tissues typically activates both embryo development and expression of the gene of interest (e.g. GUS), however, activation of the expression of the gene of interest in the absence of morphologically discernible embryos may also be observed. Expression of the gene of interest, in the absence of morphologically discernible embryos provides initial evidence for direct interaction of BNM3 with the target sequence.

Direct interaction of BNM3 with a target sequence may also be demonstrated using transient expression of BNM3 in plant protoplasts, along with the transient co-expression of an embryo-expressed promoter fused to a gene of interest (i.e. a gene of interest construct). *35S:BNM3* DNA and the gene of interest construct are introduced into protoplasts derived from non-seed cells, such as leaf mesophyll cells by electroporation. The expression of the gene of interest is examined after several hours to confirm activation of the target sequence. Direct interaction of BNM3 with the

target sequence may further be demonstrated by co-introducing the target sequence alone as competitor DNA.

In order to determine if tissues from different plant species may be transactivated by BNM3, *35S:BNM3* DNA and a reporter gene (for example, but not limited to *GUS*) construct may be introduced by microprojectile bombardment into somatic tissues of a plant. If BNM3 interacts directly with a target sequence then expression of the report er gene should coincide with transient expression of BNM3 in all species and tissues.

Direct evidence for BNM3-target sequence interaction may also be obtained by isolation of BNM3 protein expressed in bacteria, insect or yeast. *BNM3* is expressed in bacteria, insect, or yeast using commercially available expression systems and isolated to purity. Gel mobility shift assays (Gustavson *et al.*, 1991) are performed using a BNM3-target sequence, for example an embryo-expressed target sequence, to demonstrate direct binding of BNM3 to the BNM3-target sequence. Footprint analyses may also be performed to locate the region of BNM3 binding. Fragments of target sequences that bind BNM3 may then be subcloned and used as competitors for BNM3 binding in transient assays described above.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

Examples

General methods: *Microspore Embryo Culture*

Brassica napus c.v. Topas was used as the source of all plant material for microspore embryo culture. Donor plants for microspore culture were grown in a growth cabinet at 20 °C /15 °C (day/night) with a 16 h photoperiod (400 µE/m/s) provided by VHO cool white fluorescent lamps (165W, Sylvania) and incandescent bulbs (40W, Duro-test). Four weeks after germination the plants were transferred to growth cabinets under the same light conditions, but set at 10 °C /5 °C (day/night). Microspores and pollen were isolated and cultured as described in Keller *et al.* (1987), except that after 21 days in culture, cotyledon stage embryos were transferred to a maturation medium consisting of 1/2X NLN salts, 1% sucrose, 0.35 M mannitol and 5 µM ABA. Uninduced cultures (microspores and pollen continuing gametophytic development) and heat-stressed, non-embryogenic cultures (used for construction of the subtracted probe), were cultured from the same starting material as was used for the initiation of embryogenic cultures. Uninduced samples were obtained by culturing microspores and pollen for four days at 25 °C. Heat-stressed, non-embryogenic samples were obtained by culturing microspores and pollen for one day 25 °C, followed by three days 32 °C.

Samples of microspore and pollen cultured for less than 10 days were collected by centrifugation. Older samples containing globular, heart, torpedo and cotyledon stage microspore-derived embryos were collected by filtration through nylon meshes of various pore sizes as described in Ouellet *et al.* (1992). All other plant tissues were collected from greenhouse grown material. Seed material was obtained by hand pollinating flowers on the day of anthesis and collecting developing seeds on various days after pollination (DAP).

Nucleic Acid Isolation and Analysis

Total RNA was isolated using either a cesium chloride/guanidinium isothiocyanate procedure (Ouellet, 1992) or TRIZOL reagent (Gibco-BRL). RNA gel blot analysis was carried out by separation of 5 to 20 μg of total RNA per lane through 1.5% agarose gels containing 0.62 M formaldehyde, essentially as in Sambrook *et al.* (1989), followed by capillary transfer to Hybond-N nylon membranes (Amersham). Poly(A)$^+$ RNA was isolated from total RNA by oligo (dT)-cellulose chromatography (Sambrook,1989).

Genomic DNA was isolated from leaf tissue as described in Fobert *et al.* (1991) and digested with the specified restriction enzymes using standard procedures (Sambrook,1989). DNA gel blot analysis was carried out by electrophoresis of 10 μg DNA through 0.8% agarose gels followed by capillary transfer to Hybond-N membranes.

The partial 1.2 kb *BNM3A* cDNA insert was used as a probe for DNA and RNA gel blots. Hybridization to gel blots was carried out at 65 °C according to the Hybond-N protocol. The final wash conditions were 0.1X SSC, 65 °.

Subtractive Probe Construction and cDNA Library Screening

Poly (A) mRNA was isolated from late uninucleate microspores and early binucleate pollen that had been cultured for four days at 32 °C in order to induce embryogenesis (embryogenic sample) and used to synthesize first strand cDNA (Riboclone cDNA kit; Promega). The cDNA was then hybridized to a five-fold excess (by weight) of poly (A)$^+$ RNA from late uninucleate microspores and early binucleate pollen that had been cultured for one day at 25 °C, followed by three days at 32 °C to inactivate embryogenesis (non-embryogenic sample: Pechan *et al.*, 1991). The subtractive hybridization was performed essentially as described in Sambrook *et al.* (1989). The single-stranded cDNA recovered after subtraction was labelled with [α-^{32}P] dCTP using a random primers kit (BRL) and used as the subtracted probe for screening a Lambda phage cDNA library constructed from the same embryogenic

sample described above (Boutilier, 1994). Triplicate nylon filter lifts (Hybond-N) from approximately 1.5 x 10^5 plaque-forming units of the library were screened with the subtracted probe, with a random primers-labelled first strand non-embryogenic probe and with a random primers-labelled napin seed storage protein cDNA probe (pN2; (Crouch, 1983). Napin mRNAs are prevalent in the embryogenic microspore library (Boutilier, 1994) and therefore plaques hybridizing to the napin probe were removed from the subsequent screening steps. Plaques hybridizing to the subtracted probe, but not to the non-embryogenic or napin probes, were selected and subjected to two subsequent rounds of differential screening using both the subtracted and non-embryogenic cDNA probes. DNA from selected Lambda clones was isolated (Sambrook, 1989), partially digested with *Eco* RI and *Xba* I and subcloned into pGEM-4Z (Promega).

Seven cDNAs comprising 6 unique genes, one of which comprised a truncated BNM3A cDNA, were identified. Two distinct, full length BNM3 cDNA clones (BNM3A and BNM3B) were subsequently obtained by stringent screening of circa 2.5 x 10^5 plaque-forming units of a cDNA library (UniZAPII cDNA synthesis kit, Stratagene) constructed with mRNA from 10 day old globular to heart-stage microspore-derived embryos of *B. napus* c.v. Topas. The BNM3 cDNA inserts were rescued by *in vivo* excision into Blueskript SK(-) (Stratagene).

Isolation of Brassica napus Genomic DNA sequences

The Universal Genome Walker Kit (Clonetech) was used to isolate genomic DNA fragments lying upstream of the *BNM3* ATG start codon. Pools of uncloned, adaptor-ligated *Brassica napus* cv Topas genomic DNA fragments were constructed and used to isolate BNM3 genomic sequences by nested PCR. The primary PCR made use of the outer adaptor primer (AP1) supplied by the manufacturer and a BNM3 specific primer with the sequence:

5'-GAGGCAGCGGTCGGATCGTAACAGTACTCT-3' (SEQ ID NO:8).

The nested PCR made use of the nested adaptor primer (AP2) supplied by the manufacturer and a BNM3 specific primer with the sequence:

5'-CATAAGGAGAGAGAGAAAAGCCTAACCAGT - 3' (SEQ ID NO:9).

The primary PCR mixture was then diluted 1:50 and used as template for nested PCR. Both the primary and nested PCRs were performed as recommended by the manufacturer. The nested PCR products were cloned into the pGEMT-Easy vector (Promega) and sequenced. PCR products corresponding to the 5' untranslated genomic regions of both *BNM3A* and *BNM3B* cDNAs were identified.

The genomic DNA sequence spanning the *BNM*3A ATG translational start and TAG translational stop codons was isolated by PCR from *B. napus* cv Topas genomic DNA using *Pfu* polymerase (Stratagene) and the following primer combination:

5'-ACCAAGAACTCGTTAGATC-3' (SEQ ID NO:10); and
5'-AACGCATATAACTAAAGATC-3' (SEQ ID NO:11).

The primers were used under standard PCR conditions. The PCR products were cloned into the pGEMT-Easy vector and sequenced.

DNA Gel Blot Analysis and Mapping in Arabidopsis thaliana

Five hundred nanograms of arabidopsis genomic DNA (ecotypes Columbia and Landsberg *erecta*) (Shure et al., 1983) was digested with 20 different restriction endonucleases, separated by electrophoresis through 0.8% agarose gels and blotted onto Hybond N^+ nylon membrane (Amersham) using standard methods. Blots were hybridised (1.5 M NaCl, 65 °C) with a ^{32}P[dATP] random primers labelled probe (Megaprime, Amersham) corresponding to either:

1) approximately the first 405 bp of the BNM3A cDNA (SEQ ID NO.1), or

2) approximately the last 1200 nt of the *BNM3A* cDNA (SEQ ID NO.1) and then washed under conditions of low stringency (2 X SSC, 0.1% SDS at 65 °C) or moderate stringency (0.2 X SSC, 0.1% SDS at 25 °C).

A restriction fragment length polymorphism (RFLP) was identified between ecotypes Columbia and Landsberg *erecta* using the *Cfo* I restriction endonuclease. This RFLP was used to map the position of the arabidopsis BNM3 homologue on the arabidopsis genome using the Lister and Dean recombinant inbred (RI) lines (Lister and Dean, 1993). DNA from 100 recombinant inbred lines generated from a cross between ecotypes Columbia and Landsberg *erecta* was digested with *Cfo* I, transferred to Hybond N^+ nylon membrane, hybridised with the *BNM3A* cDNA (as above) and washed under conditions of low stringency. The resulting RFLP data was sent to the RI database at the Nottingham Arabidopsis Stock Centre for determination of the map location (Lander et al., 1987). The results are discussed in Example 2-2, below.

Isolation of Arabidopsis thaliana Genomic DNA sequences

Three genome equivalents of an amplified arabidopsis ecotype C24 genomic Lambda phage library (Lambda-GEM 11, Promega) were screened using a truncated *BNM3A* cDNA probe (approximately the last 1200 nt of SEQ ID NO.1). Blots were hybridised with the ^{32}P-[dATP] random primers labelled probe (as above) and then washed under conditions of low stringency (2X SSC, 0.1% SDS at 65 °C). Seven Lambda phage were initially identified. Three putative full-length Lambda phage clones containing the arabidopsis homologue of the *BNM3* gene (SEQ ID NO:6) were subsequently identified after hybridisation under conditions of low stringency with a probe derived from the 5'end of the *Brassica napus* BNM3 cDNA (nt 1-405 of SEQ ID NO1). Individual clones comprising approximately 8.0 kb of overlapping sequence were identified from each of the three phage, subcloned into pBR322 and sequenced.

Plasmid Construction for Plant Transformation

The construction of a plasmid vectors containing the *BNM3* cDNAs under control of either a *POLYUBIQUITIN* or Cauliflower Mosaic virus 35S promoter are described below. The plasmid pRAP2TUBI contains a modified *Helianthus annus POLYUBIQUITIN* promoter (Binet *et al.*, 1991) in the plasmid pRAP2T. The plasmid pRAP2T consists of the pUCAP plasmid (van Engelen *et al.*, 1995) and a nopaline synthase (nos) terminator inserted into the *Sac* 1 and *Eco* R1 restriction sites. A PCR fragment of the *POLYUBIQUITIN UbB1* promoter comprising the 5' end of the promoter to 7 bp from the 3' end of the first exon was amplified from the vector using an M13 reverse primer and the UBIQ-3' primer:

5'-CCATGGATCCAGAGACGAAGCGAAAC-3' (SEQ ID NO:12)

which includes introduced *Nco* I and *Bam* HI restriction sites. The *POLYUBIQUITIN* promoter fragment was digested with *Pst* I and *Bam* HI, gel purified and ligated into the *Pst* I and *Bam* HI sites of pRAP2T, creating the vector pRAP2TUBIHa. The full-length *BNM3B* cDNA was digested with *Eco* RI and *Xho* I restriction enzymes, blunted with Klenow enzyme, gel purified and ligated into the *Sma* I site of pRAP2TUBI making the plasmid pKB1S. An *Asc* I/*Pac* I DNA restriction fragment containing the modified *POLYUBIQUITIN* promoter, the *BNM3B* cDNA and the nos terminator was gel purified, and ligated to the *Asc* I/*Pac* I digested binary vector pBINPLUS (van Engelen *et al.*, 1995), creating the plasmid pKBBIN1S.

The construction of a vector containing the *BNM3A* cDNA under control of a double enhanced 35S promoter and AMV translational enhancer was as follows. A *Hind* III/*Xba* I DNA restriction fragment containing the double 35S promoter and the AMV translational enhancer from plasmid pBI525 (Datla et al., 1993) was ligated to *Hind* III/*Xba* I digested pRAP2T, creating the plasmid pRAP2T35S. An *Nco* I site was introduced into the *BNM3A* cDNA clone by site directed mutagenesis. The sequence of the BNM3ANCO1 primer used for mutagenesis is:

5'-ACTCCATGGATAATAACTGGTTAGGC-3' (SEQ ID NO:13).

A second primer, BNM3AHINDIII:

5' - AAATTCTCAAGCTTTGGTCCATCTTG-3' (SEQ ID NO14)

was used together with the BNM3ANCO1 primer to amplify a 305 bp fragment of the *BNM3A* cDNA. This PCR fragment was digested with *Nco* I and *Hind* III and ligated to *Nco* I/*Kpn*I cut pRAP2T35S and a *Hind* III/*Kpn* I fragment containing the region of the *BNM3A* cDNA downstream of the *Hind* III site, creating the vector p35S:BNM3. p35S:BNM3 was digested *Asc* I and *Pac* I restriction enzymes and the fragment containing the double 35S promoter, the AMV translational enhancer, the BNM3A cDNA and the nos terminator was gel purified and ligated to the *Asc* I/*Pac* I digested binary vector pBINPLUS, creating the plasmid p35S:BNM3BIN.

Both the pKBBIN1S and p35S:BNM3BIN plasmids were transferred to *Agrobacterium tumefaciens* C58C1 strain carrying the disarmed Ti plasmid pMP90 and used in transformation experiments.

Plant Transformation

Arabidopsis thaliana ecotype C24 was used as the recipient in transformation experiments. Plants were transformed using either the floral dip method described in Clough and Bent (1998) or the root transformation method described in Vergunst *et al*. (1998).

Transgenic *Brassica napus* c.v."Topas" plants were produced by *Agrobacterium tumaciens*-mediated transformation of microspore-derived embryos. Microspore-derived embryos were cultured for 5 weeks at a density of approximately 1000 embryos per ml. Overnight cultures of *Agrobacterium* were diluted 100 times in B5 medium containing 9% sucrose. Embryos were co-cultivated with the diluted bacteria for 48 hours at 24°C in darkness, with slow shaking. The embryos were then transferred to NLN13 medium supplemented with 350 mg/L cefotaxim and 200 mg/L vancomycin for at least two weeks in darkness at 25°C.

Embryos were germinated in weak light at 25°C for about 2 weeks on solid B5 medium supplemented with 2% sucrose, cefotaxim (200 mg/L) and vancomycin

(100 mg/L). Well developed hypocotyls from germinated embryos were isolated and transferred to fresh germination medium supplemented with 100 mg/L kanamycin. After two weeks on this medium, explants were subcultured to a similar medium supplemented with kanamycin (25 mg/L). Green, putative transgenic, secondary embryos become visible after one month of selection.

Microscopy

All plant material was fixed overnight at 4 °C in 0.1 M phosphate buffer pH 7.0 containing 4% paraformaldehyde. Samples were washed in 0.1 M phosphate buffer and then dehydrated in a graded ethanol series to 100% ethanol. Samples for scanning electron microscopy were critical point dried in liquid CO_2 (Balzers CPD020), and mounted on SEM stubs using conductive carbon glue. Samples were coated with 30 nm palladium/gold using a Polaron E5100 sputter coater. Samples were observed in a JEOL JSM 5200 scanning electron microscope with an acceleration voltage of 15 kV. Digital images were obtained using Orion Framegrabber. Samples for light microscopy were embedded in Technovit 7100 (Kulzer). Sections were stained for 10 seconds in 1% Toluidine blue in 1% sodiumtetraborate, rinsed with water and mounted in Euparal. Digital images were recorded using a Sony 3 CCD camera.

Regeneration Experiments

Wild-type and transgenic arabidopsis seeds were surface sterilized, plated on ½ MS media containing 20% sucrose (½MS-20) and grown at 21 °C with the plates inclined at a 60° angle. Eight wild-type seedlings and eight seedlings from each of seven independent transgenic lines were harvested 10 days after germination and separated into root, hypocotyl and leaf explants. This material was then divided into two batches. Half of the explants were continuously cultured on B5 media containing 20% glucose (B5-20). Explants were transferred to fresh B5-20 media every two weeks. The remaining explants were cultured on B5-20 containing plant growth

regulators in order to induce shoot regeneration (Vergunst *et al.*, 1998). These explants were first placed on callus inducing media (CIM; high auxin to cytokinin ratio) for two days and then transferred to shoot inducing media (SIM; high cytokinin to auxin ratio) for the remainder of the culture period. Explants were transferred to fresh SIM media every two weeks.

Example 1: Isolation and Characterization of the BNM3 Genes from Brassica napus

A subtractive screening approach was used to isolate genes preferentially expressed during the induction of *Brassica napus* c.v. Topas microspore embryogenesis (Figure 1). Two types of microspore cultures were used in the construction of a subtracted probe: embryogenic and non-embryogenic. Embryogenic cultures were obtained by subjecting late uninucleate microspores and early binucleate pollen to a 4 day, 32 ° C heat stress treatment. The non-embryogenic sample was obtained by culturing the same starting population of late uninucleate microspores and early binucleate pollen for 1 day at 25 ° C followed by 3 days at 32 ° C (Pechan *et al.*, 1991). Poly(A) mRNA was isolated from the embryogenic sample and used to synthesize first strand cDNA. The cDNA was then hybridized to an excess of poly(A)$^+$ RNA isolated from a non-embryogenic microspore/pollen sample. The non-hybridizing, single stranded cDNA, enriched for sequences present in the embryogenic sample, but absent or present at a much lower level in the non-embryogenic sample, was recovered, radioactively labelled and used as a subtracted probe for screening a cDNA library derived from the embryogenic sample described above. Plaques hybridizing to the subtracted probe, but not to a probe derived from the non-embryogenic sample, were selected and subjected to two subsequent rounds of differential screening. Seven independent cDNA clones, comprising six unique DNA sequences were found to be differentially expressed between the embryogenic and non-embryogenic samples. One of these clones, 42A1, later renamed *BNM3A* (for *Brassica napus* microspore embryo), was further characterized.

Example 2-1: The BNM3 genes encode new members of the AP2 domain class of transcriptional activators

A single *BNM3* cDNA clone, *BNM3A*, was isolated after screening an embryogenic microspore cDNA library with a subtracted probe enriched for genes expressed in embryogenic microspores and pollen. The discrepancy between the size of the cDNA clone (1.2 kb) and the size of the transcript detected on RNA gel blots (2.2 kb) indicated that this clone did not represent a full-length cDNA. Two longer cDNA clones, corresponding to the full length cDNA of the clone originally isolated, *BNM3A* (SEQ ID NO. 1), and a new clone, *BNM3B* (SEQ ID NO. 3), were isolated from a 10 day old *Brassica napus* microspore embryo cDNA library. The alignment of the DNA sequence of these clones is shown in Figure 2. The two *BNM3* cDNA clones are 2011 and 1992 nt in length, and are 97% similar at the nucleotide level, differing only slightly in the length and sequence of their 5' and 3' untranslated regions. Both cDNAs potentially encode 579 amino acid polypeptides (predicted molecular mass of 63.9 kDa, pI of 5.7) that are 97% similar at the amino acid level (Figure 3).

The genomic complexity of the *BNM3* genes was determined by hybridization of the *BNM3* cDNAs to gel blots containing *B. napus* genomic DNA (Figure 4). The *BNM3* cDNAs hybridize to two DNA fragments under high stringency conditions. The two hybridizing fragments represent the two *BNM3* genes, *BNM3A* and *BNM3B*. *B. napus* is an amphidiploid species derived from the hybridization of the diploid *B. rapa* and *B. oleracea* genomes, thus the two *BNM3* sequences are likely derived from a single copy locus in each of the parental diploid progenitors.

Search of the sequence databases indicated that the *BNM3* translation products contain two copies of an AP2 domain (Figure 3). The AP2 domain was first identified in APETALA2 (AP2), an arabidopsis protein that regulates meristem identity, floral organ specification, seedcoat development and floral homeotic gene expression (Jofuku *et al.*, 1994; WO 98/07842), and has since been identified in a wide range of

proteins with diverse functions. These functions range from the activation of genes involved in stress (Zhou, 1997; Stockinger, 1997) and ethylene response (Ohme-Takagi, 1995) to the regulation of leaf, floral and ovule development (Moose, 1996; Jofuku, 1994; Elliot, 1996; Klucher, 1996). The AP2 domain is a 56-68 amino acid repeated motif containing at least two conserved regions: a highly basic YRG element, containing a conserved YRG amino acid motif and the RAYD element. The RAYD element contains a conserved central core of 18 amino acids that is predicted to form an amphipathic a-helix, a structure that is thought to mediate protein-protein interactions. The ability of a number of AP2 domain containing proteins to bind DNA, coupled with the presence of putative nuclear localization signals and acidic regions that may function as transcriptional activators suggests these proteins function as transcription factors.

Two phylogenetically distinct classes of AP2 domain proteins, consisting of either one AP2 domain (EREBP-like) or two AP2 domains connected by a linker region (AP2-like), have been identified (Zhou, 1997). BNM3 belongs to the latter class. Search of the databases with the region corresponding to the two AP2 domains and linker region of BNM3 reveals that BNM3 is most similar to the arabidopsis AINTEGUMENTA (ANT; Elliot, 1996;Klucher, 1996) and the *Zea mays* ZMMHCF1 AP2 domain containing protein. (ZM; Daniell, 1996) Figure 5 shows an alignment of the two AP2 domains of BNM3 with those of other proteins that contain two AP2 domains. BNM3 shares 85% amino acid sequence similarity with ANT and 88% with ZMMHCF1 in this region, but only 66% amino acid similarity with AP2 and GLOSSY15 in this region. A 10 amino acid insertion in the first AP2 domain of the and BNM3 proteins further distinguishes these three proteins from other AP2 domain containing proteins (Elliot, 1996). The BNM3, AINTEGUMENTA and ZMMHCF1 proteins also share a small hydrophobic amino acid motif, LG/SFSLS, in their amino terminal regions, but otherwise show no significant similarity in their DNA or amino acid sequences outside of the AP2 domains and linker. These results indicate that the BNM3 sequences encode unique members of the AP2 domain family of proteins.

A pairwise alignment of BNM3B cDNA and amino acid, sequences with ANT or ZMMHCF-1 sequences indicated that for the BNM3B nucleotide sequence:

- there is a 56% identity with ANT cDNA (over the 1905 nucleotides of ANT) and a 58% identity with ZMMHCF1 cDNA (over the 1773 nucleotide sequence of ZM);

and for the BNM3B amino acid sequence:

- there is a 41% identity of the BNM3B protein with ANT protein (over the 555 amino acid sequence of ANT), and a 46% identity with ZMMHCF1 protein (over 485 amino acid sequence of ZM).

Example 2-2: The Brassica napus BNM3 genes are represented by a single Arabidopsis thaliana orthologue

DNA gel blot analysis of arabidopsis genomic DNA hybridised to a number of *Brassica napus BNM3A* cDNA (SEQ ID NO:1) probes under conditions of low and moderate stringency indicated the presence of a single homologue of the *Brassica napus BNM3* genes in the arabidopsis genome. An RFLP was also identified between ecotypes Columbia and Landsberg *erecta* using the *Cfo* I restriction endonuclease. This RFLP was used to map the position of the single *BNM3* homologue on the arabidopsis genome to approximately 34 cM on chromosome 5 (Lister and Dean 1993).

Screening of three genomic equivalents of an arabidopsis genomic library identified three Lambda clones containing the putative full length arabidopsis *BNM3* homologue (*AtBBM*). Sequence analysis of the three *AtBBM* clones indicated that they are identical (SEQ 1D NO:6). Figures 9A and B show respectively the restriction fragment pattern of the isolated arabidopsis genomic clones and the pattern of restriction fragments obtained after hybridisation of arabidopsis genomic DNA with the *Brassica BNM3A* cDNA probe. Comparison of the two figures indicates that the arabidopsis *AtBBM* genomic clones and the homologue identified through DNA gel blot analysis using a heterologous probe are the same. Sequence analysis of the

AtBBM genomic clones also positioned the 5' end of the *IRREGULAR XYLEM3* (*IXR3*) gene downstream of the putative *AtBBM* coding region (position 7479, Figure 9A). *IXR3* has previously been shown to map to a 150 kb region of chromosome 5 between the markers nga106 (33.26 cM) and mi438 (33.34 cM; Taylor et al., 1999). Together this data indicates that the arabidopsis orthologue of the *Brassica BNM3* genes is encoded by a single gene that maps to chromosome 5. A sequence that is very similar with *AtBBM*, TAMU BAC clone:T10B6 (accession number AP002073; Nakamura, May 18, 2000) also maps to chromosome 5.

Comparison of the structure of *Brassica BNM3A* genomic clone (SEQ ID NO:5) and the arabidopsis *AtBBM* genomic clone (SEQ ID NO:6) indicate that the predicted intron/exon boundaries are highly conserved between the two sequences. Both sequences are predicted to comprise nine exon and eight intron sequences.

Comparison of the DNA sequence of the *AtBBM* gene (SEQ ID NO:6) and the two *Brassica* cDNA sequences (SEQ ID NO:1 and SEQ ID NO:3) indicated that the three sequences are 85% similar across the entire putative protein coding region and 95% similar in the 546 nt region spanning the two AP2 domains and the linker region lying between the two AP2 domains. The nucleotide similarity to other related AP2 domain encoding genes such as *ANT* and the sequence located on clone MOE17 on chromosome 3 (accession number AB025629) in the region spanning the two AP2 domains and the linker region lying between the two AP2 domains is 76% and 78% respectively. Neither *ANT* nor the sequence on chromosome 3 shows significant DNA similarity to *AtBBM* outside of the AP2 domain encoding region.

The similarity between the predicted amino acid coding sequence of AtBBM and the two *Brassica* cDNA sequences (SEQ ID NO:1 and SEQ ID NO:3) is approximately 80% across the entire protein coding sequence and approximately 99% in the 182 amino acid region spanning the two AP2 domains and the linker region lying between the two AP2 domains. The amino acid similarity to both ANT and the AP2 domain sequence located on clone MOE17 on chromosome 3 in the amino acid

region spanning the two AP2 domains and the linker region lying between the two AP2 domains is approximately 85%. Neither of these two protein sequences shows significant similarity to AtBBM outside of the AP2 domain region of the protein.

Example 3: The BNM3 genes are preferentially expressed in developing embryos

RNA gel blot analysis (Figure 6) was used to determine the pattern of *BNM3* gene expression during microspore-derived embryo development, seed development, and in non-seed tissues. Both analyses indicate that the *BNM3* genes are preferentially expressed in developing embryos.

RNA gel blot analysis indicates that *BNM3* mRNAs are detected in microspore cultures induced to undergo embryogenesis, as well as in the subsequent globular, heart, torpedo and cotyledon stages of microspore-derived embryo development (Figure 6A). *BNM3* mRNAs are not detected in non-embryogenic microspore cultures, in freshly isolated microspores and pollen, or in microspores and pollen continuing gametophytic development in culture (Figure 6A). RNA gel blot analysis of developing seeds shows that *BNM3* expression is first detected 14 days after pollination (14 DAP), corresponding to the heart stage of embryo development. *BNM3* expression increases during the early (21 DAP) and mid-cotyledon (28 DAP) stages of embryo development and remains constant thereafter (Figure 6B). *BNM3* transcripts were not detected in any of the non-seed tissues tested, reflecting the low level or absence of transcripts in these tissues.

Example 4: Expression of BNM3 in Vegetative Tissues Promotes Asexual Embryo Formation

In order to determine the function the *Brassica napus* BNM3 proteins, the *BNM3* cDNAs were placed under the control of two separate constitutive promoter constructs, a modified sunflower *POLYUBIQUITIN* promoter construct (hereafter referred to as *UBI:BNM3*) and a double enhanced *35S* promoter construct containing

an AMV translational enhancer (hereafter referred to as 35S:*BNM3*), and introduced into arabidopsis. Analysis of the phenotype of the transformants indicates that ectopic over expression of the *BNM3* cDNAs promotes the formation of somatic embryos on vegetative structures such as cotyledons, petioles, leaf blades and the shoot apical meristem (Figure 7). The frequency of transformants producing ectopic embryos, as well as the penetrance of the ectopic embryo phenotype, was greater when the *BNM3* gene was expressed under control of the stronger double enhanced 35S promoter-AMV translational enhancer, as compared to the *POLYUBIQUITIN* promoter. Thus a high threshold level of protein product is required to increase the frequency and penetrance of the ectopic embryo phenotype.

BNM3-derived ectopic embryos contain all of the organ systems and tissue layers found in the developing zygotic embryo. *BNM3*-derived ectopic embryos are bipolar (Figures 7D and E) and consist of an axis, comprised of the hypocotyl and radicle regions, shoot and root meristems, and cotyledons (Figure 7E). In addition, each organ system contains the characteristic radial arrangement of three specialized tissue layers (epidermis, ground parenchyma and provascular tissue) found in zygotic embryos (Figure 7E). Continued expression of the *BNM3* gene within the developing ectopic embryo leads to a reiteration of the embryo-forming process, with the result that new embryos are continuously formed on the surface of pre-existing embryos (Figure 7D and E). These results provide conclusive evidence that expression of a single gene, *BNM3*, is sufficient to initiate a signal transduction cascade leading to the formation of fully differentiated asexually-derived embryos.

Example 5: Expression of BNM3 Increases the Regeneration Capacity of Plant Tissues

We examined the effect of *BNM3* gene expression on the ability of arabidopsis plants to regenerate shoots *in vitro* in the presence or absence of added growth regulators. Leaf, root and hypocotyl explants from 10 day old seedlings of wild-type arabidopsis and transgenic arabidopsis lines expressing *BNM3* under control of the

POLYUBIQUITIN promoter were placed on media containing growth regulators to induce first callus formation and then shoot organogenesis. Root explants from transgenic lines show at least a 5-fold increase in shoot regeneration in the presence of hormones as compared to wild-type root explants. (Figure 8A). These shoots also developed faster in the transgenic explants as compared to the wild-type. Wild-type leaf and hypocotyl explants responded by producing callus on the cut end of the petiole (Figure 8B). In contrast, explants from transgenic lines immediately produced new shoots (Figure 8B) or roots from the cut end of the petiole. Transgenic explants that initially produced roots eventually also produced shoots.

Transgenic explants were also able to regenerate in absence of added growth regulators. Wild-type leaf and hypocotyl explants placed on medium lacking growth regulators occasionally produced callus or roots at the cut end of the leaf petiole, however shoots did not regenerate from these structures (Figure 8C,D). Wild-type roots greened and formed thickened nodule-like structures at the junction with lateral roots, but did not develop further. In contrast, transgenic explants placed on media lacking growth regulators regenerated shoots either from the cut end of the leaf and hypocotyl explants or from the nodule-like structures of root explants (Figure 8C,D).

All citations are herein incorporated by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

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